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Inhibition of protein kinases C prevents murine cytomegalovirus replication

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For successful establishment of infection and initiation of the replication cycle, murine cytomegalovirus (MCMV) utilizes cellular structures and functions, including cell-membrane penetration, capsid dismantling and cytosolic transport of viral DNA into the nucleus. These early events of MCMV infections are dependent on cellular regulatory mechanisms, primarily protein phosphorylation. In the present study, protein kinase inhibitors were used to explore the role of protein phosphorylation mediated by protein kinases C (PKCs) in the very early events of MCMV infection. Inhibitory effects were determined by immunofluorescence and Western blot analysis of MCMV IE1 and E1 protein expression and by production of infectious virions in cell culture. It was found that H-7, a broadly specific inhibitor of cellular protein kinases, prevented virus replication in a dose-dependent and reversible manner, and that the block in replication occurred very early in infection. More specific PKC inhibitors (sangivamycin, calphostin C and bisindolylmaleimide II), Ca^{2+}/calmodulin inhibitors (EDTA and W7) and phorbol esters (PMA) were used to dissect PKC-subclass contribution in the very early events of MCMV replication. The results indicate that the role of diacylglycerol/phorbol ester-dependent but calcium-independent PKCs is essential for establishment of MCMV infection in the host cell, starting at a very early stage of infection.

INTRODUCTION

Entry into the host cell is of fundamental importance in infection and pathogenesis for all viruses. Early events in viral infection include attachment of the virion to the cell surface, penetration through the cell membrane, unpacking of capsids and transport of viral genomes to a site where viral DNA replication takes place. Successful completion of this process is a prerequisite for initiation of the infection cycle.

Murine cytomegalovirus (MCMV) is a betaherpesvirus that establishes productive cytolytic or non-productive latent infection in cells. The MCMV virion consists of an icosahedral protein capsid surrounding the double-stranded DNA genome, which has to be released into the cytoplasm (reviewed by Lučin & Jonjić, 1995). The entry route of MCMV and the very early events of its transport into the nucleus are still poorly understood. Various pathways are used by herpesviruses to penetrate host cells. Membrane fusion is the most common process, occurring either at the cell plasma membrane or within endocytic vesicles (reviewed by Smith & Helenius, 2004). Among herpesviruses, differences in entry are observed, depending not only on the type of virus, but also on the targeted cell type (Miller & Hutt-Fletcher, 1992). Human cytomegalovirus (HCMV) enters the cell by pH-independent fusion of the viral envelope with the plasma membrane, which is initiated by a signal generated inside the glycoprotein B cytosolic domain after binding to the cellular receptor (Tugizov *et al.*, 1999). Other events after the entry of betaherpesviruses, prior to the initiation of gene expression, require triggering of a signalling cascade that allows local reorganization of the cytoskeleton and propagation of viral components through the cytosol. It has been reported that the cellular protein kinases C (PKCs) are required in the early phase of influenza virus entry into the cell nucleus (Root *et al.*, 2000) and, in later phases, for herpesvirus nucleocapsid assembly and egress from the nucleus (Mettenleiter, 2002; Muranyi *et al.*, 2002).

Protein kinases are key intracellular elements of signal-transduction pathways that control gene expression, modulate metabolic processes and allow the cell to respond to constantly changing extracellular conditions or to intracellular modulations induced by intracellular microbial agents (Krajcsi & Wold, 1998). The PKCs comprise a large superfamily of related proteins that carry out diverse regulatory roles in many cellular processes (Newton, 1997). Based on structural variations and biochemical properties, the PKC family of proteins can be categorized into three groups: classical (cPKC), novel (nPKC) and atypical (aPKC). cPKCs are calcium- and phospholipid-dependent, whereas nPKCs and aPKCs do not require any calcium for their activities (Newton, 1997). Activation of the cPKC and nPKC isoforms typically involves recruitment to membranes and

interaction with both phosphatidylserine and the second messenger diacylglycerol (DAG). Tumour-promoting phorbol esters can activate both cPKCs and nPKCs, whereas aPKCs are insensitive to phorbol esters and DAG (Way *et al.*, 2000).

Small molecules that act by inhibition of novel molecular targets, such as the cellular and viral protein kinases, are currently being considered as novel therapeutic candidates against herpesviruses (Wathen, 2002). Among the small-molecule kinase-inhibitor candidates, the H-series isoquinolinesulfonamide inhibitors (H-7, H-8 and H-89) inhibit protein kinase activity by competitively inhibiting ATP interactions with eukaryotic kinases (Hidaka *et al.*, 1984). Within the group of H-series inhibitors, H-7 is known to be non-selective in its action and inhibits a wide range of different protein kinases (Quick *et al.*, 1992). In addition to the H-series of PKC inhibitors, staurosporine (Ward & O'Brian, 1992) is a non-specific kinase inhibitor that inhibits both Ca^{2+}-dependent and -independent PKCs at the conserved site in the catalytic domain. Sangivamycin, a potent inhibitor of PKCs, competes with the binding of ATP to the catalytic fragment (Loomis & Bell, 1988). Calphostin C (CC) (Kobayashi *et al.*, 1989; Tamaoki & Nakano, 1990), an inhibitor of the classical and novel PKC isoforms, and bisindolylmaleimide II (BIM II) (Toullec *et al.*, 1991), an inhibitor of different PKC isozymes, are highly potent and specific inhibitors of PKCs. The inhibitory potential of BIM II is dependent on concentration, showing more specificity and selectivity for members of the cPKC family at the nanomolar range and to the nPKCs at the micromolar range (Basu, 1998; Wilkinson *et al.*, 1993).

In the present study, we used the specified protein kinase inhibitors to explore the role of protein phosphorylation mediated by PKCs in the very early events of MCMV infection, prior to initiation of the replication cycle and viral gene expression. We have found that virus replication was inhibited by these inhibitors in a dose-dependent and reversible manner, and that the block occurred at very early stages of infection.

METHODS

Cells, virus and infection conditions. Primary murine embryonic fibroblasts (MEFs) were generated from BALB/c mice and propagated in minimal essential medium (MEM) supplemented with 5 % (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 μg streptomycin ml^{-1} and 100 U penicillin ml^{-1} (all from Gibco). The cells were grown as an adherent monolayer in Petri dishes and used for infection after three *in vitro* passages when they were 90 % confluent. The wild-type MCMV of the Smith strain (VR-194; ATCC) was propagated on third-passage BALB/c MEFs and purified by sucrose-gradient centrifugation. MEFs were infected with 0·1 p.f.u. virus per cell by using the centrifugal enhancement of infectivity technique at 800 ***g*** for 30 min.

Reagents and antibodies. H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride] (10–50 μM), phorbol 12-myristate 13-acetate (PMA) (10 nM), CC (50 nM to 0·5 μM), BIM II (50 nM to 10 μM), sangivamycin (10–50 μM), EDTA (1·5 mM), [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (W7) (10 μM) and a mAb against α-tubulin (clone DM 1A) were provided by Sigma-Aldrich. mAbs *croma-101* (anti-IE1) and *croma-103* (anti-E1), produced in our laboratory, were used as tissue-culture supernatant or semipurified ascites.

Immunofluorescence. MEFs, grown in six-well tissue-culture plates on glass coverslips, were used for immunofluorescence experiments. Before staining, they were washed with PBS, permeabilized with ice-cold methanol, blocked with PBS containing 2 % FCS and incubated with mAbs diluted in PBS containing 2 % FCS and 0·3 % NaN_3. Bound antibodies were visualized by fluoresceinated goat anti-mouse IgG (Becton Dickinson). After immunofluorescence, cells were stained by Evans blue (red fluorescence of the cytoplasm) and analysed by fluorescent microscopy.

Metabolic labelling and immunoprecipitation. Cells were incubated for 30 min at 37 °C in methionine-free MEM (Gibco) and labelled with [^{35}S]methionine (Amersham Biosciences) at 200 μCi (7·4 MBq) ml^{-1}. After labelling, cell monolayers were washed three times with cold PBS and lysed in a buffer containing 10 mM Tris/HCl (pH 7·6), 150 mM NaCl, 1 % SDS, 1 % sodium deoxycholate and 1 mM PMSF. After centrifugation at 14 000 r.p.m. for 20 min, the cellular extracts were used for immunoprecipitation. Cellular lysates were precleared with 50 % protein A–Sepharose slurry (Amersham Biosciences) at 4 °C for 30 min. Immunoprecipitation of the immediate-early 1 (IE1) and early 1 (E1) viral proteins was performed with ascitic fluid of mAbs *croma-101* and *croma-103* (3 μl each). Immune complexes were retrieved with protein A–Sepharose (50 μl of 50 % slurry) at 4 °C for 1 h, eluted at 96 °C by incubation with SDS sample buffer [0·125 M Tris/HCl (pH 6·8), 20 % glycerol, 3 % SDS, 2 % β-mercaptoethanol, 0·05 % bromphenol blue] and analysed by SDS-PAGE (10 % gel) under reducing conditions. Polyacrylamide gels with metabolically labelled proteins were exposed to autoradiography film containing scintillating emulsion (Biomax MR; Kodak).

Western blot analysis. Cellular extracts for Western blot analysis were prepared in SDS sample buffer, separated by reducing SDS-PAGE and blotted onto a PVDF Western blotting membrane (Roche Diagnostics) at 60–70 V for 2 h. PVDF membranes were washed in Tris/HCl-buffered saline (TBS) (50 mM, pH 7·5) and incubated in 1 % blocking reagent (Roche Diagnostics) for 2 h, followed by 1 h incubation with mAbs (1 : 500 dilution of ascitic fluid), three cycles of washing (TBS with 0·1 % Tween 20; TBS-T buffer) and 45 min incubation with peroxidase-conjugated goat anti-mouse Ig antibody (1 : 1000 dilution) in TBS buffer containing 0·5 % blocking reagent. After washing with TBS-T buffer, immunocomplexes were visualized by using a substrate solution containing 0·013 mM diaminobenzidine, 0·02 % H_2O_2 and 0·03 % NiCl/$CoCl_2$ in PBS. For a negative control, Western blots of MCMV-infected cells were probed with isotype-mached irrelevant mAbs (IgG1 and IgG2a) (Jackson ImmunoResearch Laboratories). Signals of bands on Western blot membranes were quantified with a calibrated imaging densitometer (GS-710; Bio-Rad).

Virus-titre assays. MEFs were treated for 1 h with inhibitors, dissolved in MEM containing 5 % FCS or appropriate vehicles (PBS, DMSO, ethanol) used for preparation of the inhibitor, and infected with MCMV at an m.o.i. of 10 by using the centrifugal enhancement of infectivity technique at 800 ***g*** for 30 min. Thirty hours after infection, cell cultures were subjected to three cycles of freezing and thawing to release infectious virus particles from cells. Number of infectious particles in tissue-culture homogenates was determined by titration on monolayers of subconfluent MEF cultures in 48-well plates by the standard viral-plaque assay, as described previously

(Lučin *et al.*, 1994). Titration was carried out in four replicates and results were presented as number of infectious virions (p.f.u.) and compared by using the Mann–Whitney *U* test.

RESULTS

H-7 blocks MCMV replication at an early stage of infection

A wide-range protein kinase inhibitor, H-7, was used to study requirements for protein phosphorylation during early stages of MCMV infection. We infected MEFs with MCMV and treated cells with the inhibitor 1 h prior to infection. Infected cells were assayed by Western blotting and by immunofluorescence microscopy at 3 h post-infection (p.i.) by using antibodies specific for the MCMV IE1 (76/89 kDa) and E1 (36/38 kDa) proteins.

Expression of the IE1 protein could be detected by immunoprecipitation after 30 min and reached a maximum between the first and third hours after infection (Fig. 1a). At 2 h p.i., IE1 could be detected by Western blotting (not shown) and, at 3 h p.i., it could be detected by immunofluorescence, showing a diffuse nuclear staining except in distinct areas of the nucleus (Fig. 1b). The E1 protein is expressed later, as a product of the first set of early genes (Bühler *et al.*, 1990). It is expressed throughout the early phase (Fig. 1a) and it can be visualized in the nucleus at 3 h p.i. by immunofluorescence, with a characteristic speckled distribution (Fig. 1b).

Western blot analysis of infected cells that were pre-treated with increasing concentrations of H-7 demonstrated a decrease in expression of the IE1 (Fig. 2a) and E1 (data not shown) proteins. However, data from Western blotting show that treatment of cells with H-7 results in inhibition of IE1 (and E1) expression from the population of infected cells (Fig. 2a). To examine this in more detail, IE1 (and E1) expression was also examined by immunofluorescence. This showed that, whilst IE1 (and E1) was undetectable in most cells, a small proportion of cells expressed similar levels to those seen during infection in the absence of inhibitor (Fig. 2b). Thus, the low level of IE1 seen on Western blotting of samples from cells infected in the presence of H-7 can be ascribed to normal expression of IE1 by a small proportion of cells, rather than to uniform inhibition of expression in all infected cells. By decreasing the concentration of the inhibitor, the proportion of IE1 (and E1)-positive cells by immunofluorescence increased (data not shown). These data show that H-7 prevents expression of the IE1 and E1 MCMV proteins in a dose-dependent manner and indicate that the inhibitory effect is active at a very early stage of infection, prior to transcription of immediate-early genes.

To examine the overall effect of H-7 on the production of infectious virions, we treated infected cells with 50 μM inhibitor 1 h prior to infection or 4 and 18 h after infection, or left them untreated. When cells were infected with a high infectious load (m.o.i. of 10), untreated fibroblasts developed cytopathogenic effect (not shown) and produced high numbers of infectious virions 30 h after infection, whereas cells treated with H-7 prior to or 4 h after infection showed very little cytopathogenic effect (not shown) and reduced assembly of infectious virions significantly (Fig. 2c). Although cytopathogenic effect was present in cells when H-7 was added 18 h p.i., the formation of infectious virions was reduced significantly (Fig. 2c). These data confirm that H-7 has a significant inhibitory effect on MCMV replication, even if it was added at later stages of the MCMV replication cycle. In addition, these data indicate that targets for the inhibitory activity of H-7 were active in all phases of the MCMV replication cycle and essential for progression of the MCMV replication programme. They are required for steps prior to MCMV gene expression, for modification of cellular functions during the early phase (e.g. cell rounding and major histocompatibility complex class I downregulation; N. Kučić, unpublished data) and for virion assembly and release from infected cells at the end of the late phase.

H-7 reversibly blocks entry of MCMV into the nucleus

Our previous data indicated that the inhibitory effects of H-7 on MCMV replication occur at very early times of infection. To test the inhibitory effects on virus entry into the cell, we treated fibroblasts with H-7 and infected them with MCMV. Two hours after infection, H-7 was washed out and, 3 h later, the cells were examined by

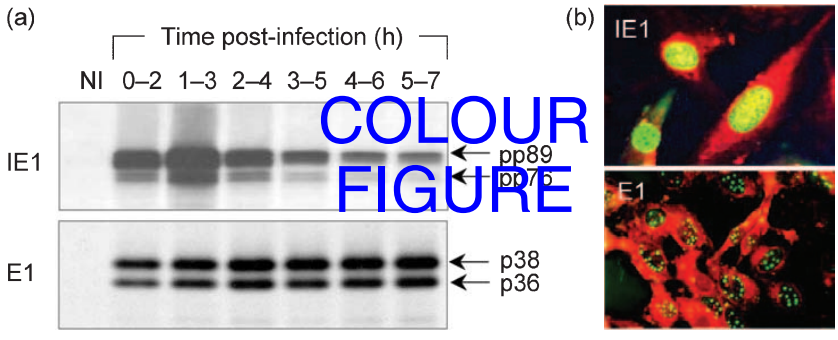

Fig. 1. Expression kinetics and cellular localization of IE1 and E1 MCMV proteins. (a) MEFs were infected with MCMV and pulse-labelled with [^{35}S]methionine for 2 h throughout the early phase of the MCMV replication cycle. IE1 and E1 proteins were immunoprecipitated from lysates and separated by SDS-PAGE. (b) Cellular localization of IE1 (pp89/76) and E1 (p38/36) proteins in infected fibroblasts at 3 h p.i., as determined by indirect immunofluorescence (green fluorescence). Cell cytoplasm was stained with Evans blue (red fluorescence).

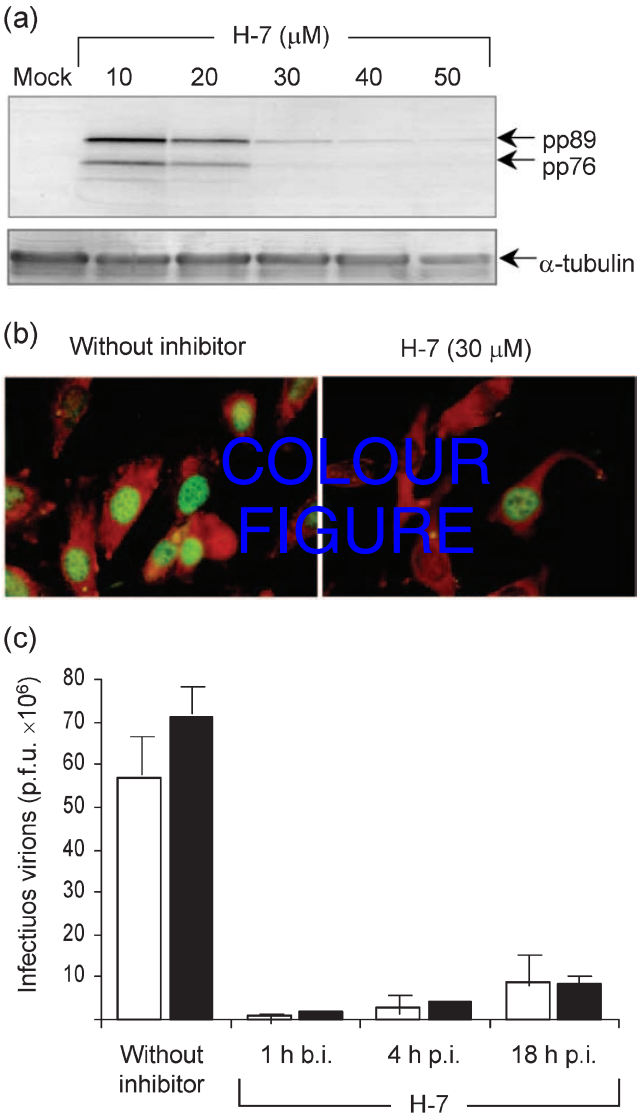

Fig. 2. H-7 inhibits IE1 expression and formation of infectious virions. (a) MEFs were treated with various concentrations of H-7 1 h before infection with MCMV and, 3 h after infection, expression of the IE1 protein (pp89/76) was analysed by immunoblotting. Cellular protein load was determined by staining of α-tubulin. (b) Expression of IE1 in cells that escaped the effect of H-7, as determined by immunofluorescence. (c) MEFs were treated with 50 μM H-7 and infected with MCMV. H-7 was added 1 h before infection (b.i.), 4 h after infection (p.i.) or 18 h p.i. Infectious virions in tissue-culture homogenates were determined 30 h p.i. Mean values of two independent experiments (empty bars, experiment 1; filled bars, experiment 2) and SEM of four replicates are shown.

immunofluorescence. IE1 and E1 expression after washout was similar to that in cells that were infected in the absence of inhibitor, whereas it was almost completely absent in the presence of H-7 (Fig. 3). These data indicate that H-7 reversibly blocks entry of MCMV into the cell or unpacking of the nucleocapsid and its transport into the nucleus.

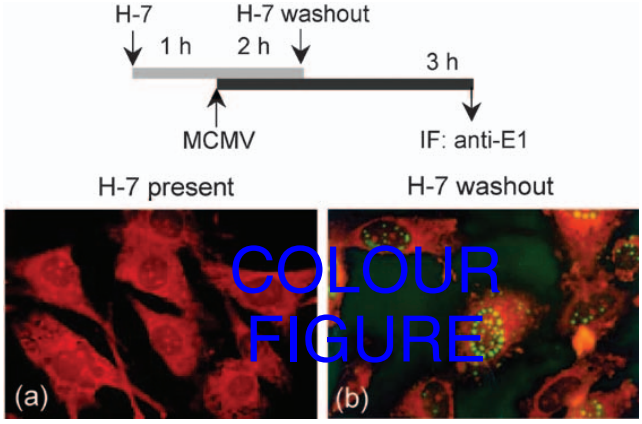

Fig. 3. H-7 reversibly blocks progression of the very early phase of MCMV infection. MEFs were treated with 50 μM H-7 and infected with MCMV. H-7 was added 1 h prior to infection and was continuously present in the cell culture during the whole experiment (a) or was removed by washing out from the culture medium 1 h after infection (b). Three hours after infection, the E1 protein was determined by immunofluorescence (IF).

PKC activity is required for progression of the very early phase of MCMV replication

To dissect further the inhibitory effects of H-7, we treated cells with a panel of PKC inhibitors: BIM II (Davis *et al.*, 1992; Wilkinson *et al.*, 1993), sangivamycin (Loomis & Bell, 1988) and CC (Kobayashi *et al.*, 1989; Tamaoki & Nakano, 1990; Redman *et al.*, 1995). These inhibitors are not entirely selective, but their differences in specificity and molecular targets may help to understand and localize the principal actors during the early stages of replication.

MEFs were treated with selected inhibitors and the expression of IE1 and E1 was determined 3 h after infection by immunofluorescence and Western blotting. BIM II, tested in nanomolar and micromolar concentrations, showed little inhibitory effect on the expression of IE1 (Fig. 4a) and E1 (not shown). At the 10 μM concentration of BIM II, a large proportion of cells expressed IE1 and E1, whilst increasing its concentration enhanced its inhibitory action, but also its toxic effect on cells (data not shown). In contrast, sangivamycin (Fig. 4a) and CC (Fig. 4b) blocked IE1 and E1 expression completely at micromolar concentrations.

Protein-expression data could be confirmed by the experiment showing formation of infectious virions, which indicates that sangivamycin and CC are potent inhibitors of the formation of infectious virions in treated cells (Fig. 5). At nanomolar concentrations, BIM II did not inhibit formation of infectious virions, whereas increasing its concentration to the micromolar range resulted in an increased inhibitory effect (Fig. 5a), reducing infectious-virion formation to one-third of the control value. These data were reproduced in three independent experiments (Fig. 5b).

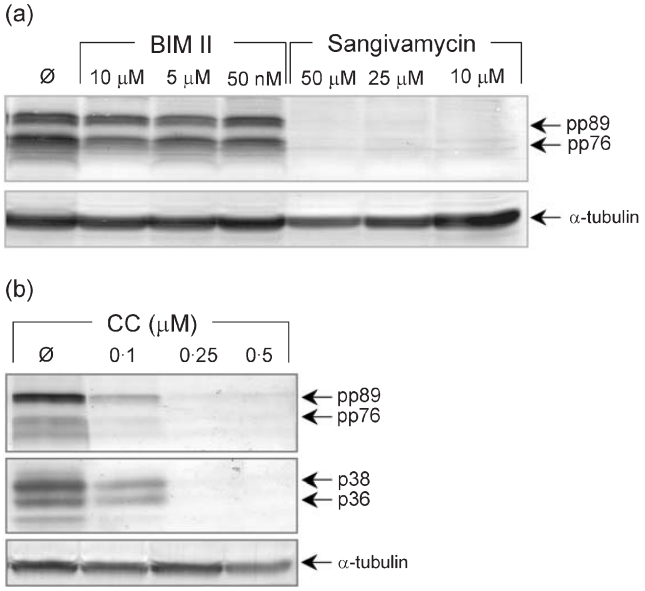

Fig. 4. Effects of PKC inhibitors on the expression of IE1 and E1 proteins. MEFs were left untreated (Ø) or treated with the indicated concentrations of (a) BIM II, sangivamycin or (b) CC 1 h before infection with MCMV. Three hours after infection, the expression of IE1 (pp89/76), E1 (p38/36) and cellular α-tubulin was determined by Western blot analysis.

Thus, we can conclude that active PKCs are essential for progression of very early stages of MCMV infection, as BIM II and CC, potent and specific inhibitors of PKCs, lead to a decrease in infectious-virion formation.

Dissection of PKC-subclass requirement in the very early phase of MCMV replication

The concentration-dependent inhibitory effect of BIM II (Fig. 5a) indicates for its action on nPKCs (Wilkinson *et al.*, 1993; Basu, 1998), and the inhibitory effect of CC (Fig. 5b) for involvement of DAG/phorbol ester-sensitive PKCs (Redman *et al.*, 1995). Phorbol esters, such as PMA, act as PKC agonists (Castagna *et al.*, 1982; Redman *et al.*, 1995) and could activate the 'classical' and 'novel' subclasses of PKCs, but not 'atypical' PKCs that are unresponsive to Ca^{2+} and phorbol esters. To examine the effect of PKC stimulation on the very early phase of MCMV replication, we pre-treated infected fibroblasts with PMA and determined the expression of IE1 and E1 proteins by Western blot and immunofluorescence, as well as formation of infectious virions. Western blot analysis demonstrated that treatment of cells with PMA resulted in increased expression of the IE1 and E1 proteins (Fig. 6a). The increase in amount of the IE1 and E1 proteins was proportional, as estimated by quantification of relative intensities of bands on the Western blot membrane, compared with the α-tubulin control (Fig. 6a). The effect of PMA in immunofluorescence appeared as an increase in the fluorescence intensity within an individual cell and as an increase in the percentage of IE1 (and E1)-positive cells (Fig. 6b). The enhancement of MCMV infection by PMA treatment was confirmed by a virion-production assay. Fig. 6(c) demonstrates two experiments in which the production of infectious virions after PMA treatment was seven- to ninefold higher then in the control. The effect achieved with PMA could be abrogated by addition of the PKC inhibitor CC (data not shown). These data indicate that stimulation of PKCs, which are responsive to phorbol esters, may be favourable for viral protein expression by enhancement of the very early events of MCMV replication.

To distinguish which subclass of PKC is required for the early phase of infection with MCMV, prior to infection, we treated cells with EDTA to deplete extracellular Ca^{2+} and with W7 to antagonize calmodulin, an intracellular calcium-binding protein (Inagaki *et al.*, 1983), and compared their effect with inhibitory concentrations of BIM II and H-7. Both inhibitors, EDTA and W7, applied at the same time (Fig. 6a) or separately (not shown), had no effect on IE1 or

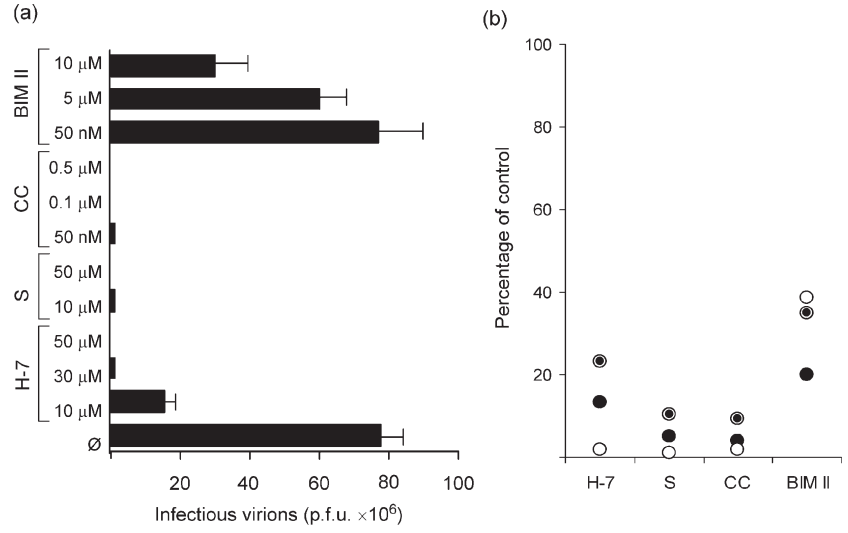

Fig. 5. Effects of PKC inhibitors on production of infectious virions. (a) MEFs were left untreated (Ø) or treated with indicated concentrations of H-7, sangivamycin (S), CC or BIM II and infected with MCMV. Infectious virions in tissue cultures were determined 30 h after infection by plaque assay. Mean values and SEM of four replicates are shown. (b) Production of infection virions in three independent experiments, expressed as percentage of mock-treated control (concentrations of inhibitors used: 10 μM H-7, 10 μM S, 50 nM CC and 10 μM BIM II). Each point represents the mean value of four replicates from each experiment (black dots, experiment 1; shaded dots, experiment 2; white dots, experiment 3).

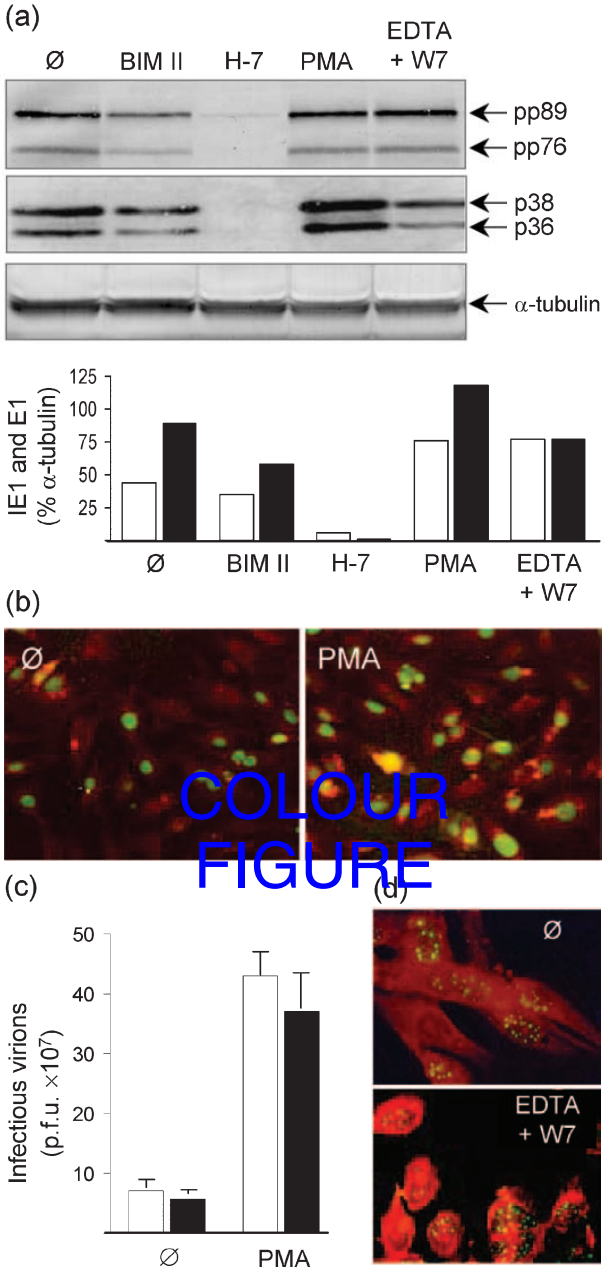

Fig. 6. The effect of PMA and EDTA/W7 on MCMV infection. (a) MEFs were treated with BIM II (10 μM), H-7 (10 μM), PMA (10 nM) or EDTA (1·5 mM) and W7 (10 μM) 1 h before infection with MCMV. Three hours after infection, the expression of IE1 (empty bars; pp89/76), E1 (filled bars; p38/36) and cellular α-tubulin was determined by Western blot analysis. Results of densitometric analysis are represented as a percentage of the relative intensity of bands on the Western blot compared to the α-tubulin control. (b) Increased proportion of cells expressing IE1 protein in cells treated with PMA (10 nM), compared with untreated control (Ø). (c) Formation of infectious virions in tissue cultures (10 nM PMA), determined 30 h after infection by plaque assay. Mean values of two independent experiments and SEM of four replicates are shown. (d) Immunofluorescence staining of E1 and the cellular shape 3 h after infection in untreated cells (Ø) and cells pre-treated with EDTA/W7.

E1 expression, indicating that intracellular Ca^{2+} is not required for virus entry into the cells and entrance into the replication cycle, although the effect of the inhibitors on cellular shape and attachment was evident at 3 h p.i. (Fig. 6d). Indirectly, we can conclude that PKC inhibitors exert their inhibitory effects by acting predominantly on 'novel' PKCs, rather than on cPKCs.

DISCUSSION

We have shown that PKC inhibitors arrest progression of MCMV infection at very early stages, but they also act in later phases, indicating that protein kinases are required throughout the replication cycle of the virus. PKCs are a large superfamily of related proteins that carry out diverse regulatory roles in various cellular processes (Parekh *et al.*, 2000; Toker, 1998) and these processes are taken over by the virus (Constantinescu *et al.*, 1991; Krajcsi & Wold, 1998). Specific inhibitors for various isoforms are not available and, therefore, cannot be used to dissect their role. Pharmacological probes for the action of PKC-family proteins include membrane-permeable allosteric activators (e.g. phorbol esters) and catalytic inhibitors (e.g. H-7, sangivamycin and staurosporine). In general, these do not distinguish isoforms and, furthermore, they can modulate other C1 domain-containing proteins (e.g. phorbol esters) or protein kinases (catalytic-site inhibitors). However, their combined use is a helpful guide to PKC involvement in cellular processes (Parker & Murray-Rust, 2004; Way *et al.*, 2000). In this report, we used a panel of inhibitors with different inhibitory mechanisms in order to dissect PKC-subfamily requirement for progression of very early events of MCMV infection.

H-7, a competitive inhibitor of the ATP-binding site at the catalytic domain of a protein kinase (Hidaka *et al.*, 1984), prevented MCMV infection in a dose-dependent and reversible manner. H-7 and other isoquinolinesulfonamide inhibitors, such as H-8 and H-89, are inhibitors of a wide range of protein kinases, including PKC, PKA and PKG (Engh *et al.*, 1996). H-89 has a similar inhibitory effect to H-7 (N. Kučić, unpublished data), although H-89 shows the highest specificity for PKA (Hidaka & Kobayashi, 1992). Besides H-7, sangivamycin, a wide-range protein kinase inhibitor (Loomis & Bell, 1988), showed almost-complete inhibition of MCMV infection, starting at very early stages, which resulted in almost-complete inhibition of virion assembly and release of infectious virions. Sangivamycin competes with binding of ATP to the catalytic fragment of PKC and thus inhibits the PKC family (Loomis & Bell, 1988). Distinct from H-7, an additional inhibitory effect of sangivamycin is attributed to competition with the phosphatidylserine-binding site, and it does not exert its action through the lipid-binding/regulatory domain (Loomis & Bell, 1988).

Furthermore, we compared the effects of two more specific PKC inhibitors that exhibit differential specificity toward

PKC isozymes: CC, a highly potent and specific inhibitor of DAG/phorbol ester-sensitive PKCs (Kobayashi *et al.*, 1989; Tamaoki & Nakano, 1990) that acts by inhibition of phorbol dibutyrate and other phorbol esters binding to PKC by competition with DAG (Redman *et al.*, 1995), and BIM II, an inhibitor with isozyme specificity for cPKCs and nPKCs (Toullec *et al.*, 1991). CC, which inhibits the classical and novel PKC isoforms (Tamaoki & Nakano, 1990), completely prevented IE1/E1 expression and formation of infectious virions at submicromolar concentrations. In contrast, BIM II had a partial inhibitory effect on MCMV replication only at micromolar concentrations, whilst in a nanomolar range, it had no effect at all. It has been reported that the specificity of BIM II on types of PKCs is concentration-dependent (Basu, 1998). cPKCs are the most sensitive to BIM II, for which IC_{50} values are in the nanomolar range, whilst nPKC can be inhibited by micromolar concentrations (Basu, 1998; Wilkinson *et al.*, 1993). Thus, our experiments with BIM II indicate the involvement of nPKCs, rather than cPKCs, in early events of MCMV infection. In addition, within the PKC-activation cascade at the plasma membrane, BIM II acts at the end, whereas CC acts at the beginning of recruitment and phosphorylation reactions of PKC that are required to generate the 'mature', phosphorylated form (Parekh *et al.*, 2000), where a BIM II-sensitive molecule amplifies the signal from the CC-sensitive molecule. This can be an explanation for the stronger inhibitory activity of CC, especially if it is known that the effects of PKC phosphorylation are concerned primarily with its intrinsic catalytic activity and ability to phosphorylate and modify the actions of its own downstream targets. Enhancing effects of PMA support the evidence for involvement of the classical and novel subclasses of PKC, which have a phorbol ester-binding site and could be stimulated by PMA. In addition, the absence of inhibitory effect after cytoplasmic Ca^{2+} depletion by the calcium chelator EDTA and inhibition of calmodulin by W7 (O'Brian & Ward, 1989) indicates the involvement of PKCs that are not stimulated by or dependent on Ca^{2+} ions. cPKCs are calcium- and phospholipid-dependent, whereas nPKCs and aPKCs do not require any calcium for their activities (Newton, 1997). Therefore, our results suggest the role of DAG/phorbol ester-dependent, but calcium-independent, PKC during the early stage of MCMV infection.

All PKC inhibitors used in this study have different mechanisms of action and their inhibitory effects are concentration-dependent. Reduction of their concentration did not result in uniform increase of IE1 and E1 expression in all cells, but rather with an increase in the proportion of cells that expressed IE1 and E1 proteins to the level achieved in the absence of inhibitor. Thus, it seems that cells that escape the effect of an inhibitor express IE1 and E1 almost to normal levels. As the cells cannot escape exposure to the solubilized inhibitor, the outcome for an individual cell must be determined by the number of viral particles that interact successfully with that cell. This may indicate that the inhibitory activity is an 'all or nothing' effect (Root *et al.*, 2000), suggesting a specific early block: those virions that escape drug inhibition go on to give normal levels of protein expression.

It is known that preceding activation of PKC induces a considerable increase in HCMV infection of endothelial cells and that activity of PKC is modulating factor for HCMV infectivity (Slobbe-van Drunen *et al.*, 1997). The mechanism by which HCMV and MCMV enter the cell is unclear, but it is known that the early events in response to HCMV, including phosphorylation of the cell-surface glycoprotein H receptor, is mediated by tyrosine kinases rather than PKCs (Keay & Baldwin, 1996). This may indicate that cell-phosphorylation processes mediated by PKCs are involved in steps of viral transport to the nucleus and viral protein expression after virion internalization. Several groups have suggested a role for PKC in endocytosis, as well as for virus entry into the cell (Constantinescu *et al.*, 1991; Root *et al.*, 2000; Siecskarski & Whittaker, 2002). The entry of several enveloped viruses, including rhabdoviruses, alphaviruses, poxviruses and herpesviruses, has been proposed to require PKC for the processes of viral movement through the cell. These results have been based on the action of protein kinase inhibitors, such as H-7 and staurosporine (Constantinescu *et al.*, 1991). More recently, it has been shown that the successful entry of influenza virus requires PKC (Root *et al.*, 2000). In the presence of BIM I, influenza virus is prevented from entering the cell or from escaping endosomes and accumulates in cytoplasmic vesicles near the cell periphery. Furthermore, the role of PKC has been confirmed in herpesvirus nucleocapsid egress from the cell nucleus during the enveloping process (Muranyi *et al.*, 2002).

Cellular protein kinases could be required for remodelling of the cellular cytoskeletal network. The size and structural organization of the cytoskeleton itself would provide a serious obstacle to the diffusion of virus nucleocapsid, viral protein complexes and even individual proteins in the absence of regulated and facilitated transport. The composition of the cytoskeleton makes free diffusion minimal. Thus, by utilizing the trafficking network of the cytoskeleton, virus and its components would traverse quickly through the cytoplasm to direct their perinuclear accumulation (reviewed by Campbell & Hope, 2003). The efficient transport system of the eukaryotic cell is mainly composed of actin microfilaments, which are used for short-range transport, and microtubules, which are used for long-range transport that is partially regulated by phosphorylation mediated by PKC (Keenan & Kelleher, 1998). The actin network is required primarily for virion entry, and microtubules for active transport of viral proteins to the nuclear membrane (Campbell & Hope, 2003). Disruption of actin and microtubular networks by cytochalasin D and nocodazole synergistically prevented expression of the IE1 and E1 proteins in our model (N. Kučić, unpublished data). Similarly, it has been demonstrated that intact microtubules are required for the infection of epithelial cells with adenovirus and herpes simplex virus (Sodeik *et al.*, 1997).

In the past, the search for antiviral drugs was focused mainly on replicases and other viral enzymes. One of the most effective ways of preventing virus infection and disease is to prevent the initial entry of virions into their target cells or very early events during infection. Thus, further studies on very early events of virus replication will add to our knowledge of virus entry and transport to the cell nucleus, as well as understanding the contribution of cellular mechanisms and signalling pathways.

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REFERENCES

Basu, A. (1998). The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-α. *Mol Pharmacol* **53**, 105–111.

Bühler, B., Keil, G. M., Weiland, F. & Koszinowski, U. H. (1990). Characterization of the murine cytomegalovirus early transcription unit e1 that is induced by immediate-early proteins. *J Virol* **64**, 1907–1919.

Campbell, E. M. & Hope, T. J. (2003). Role of the cytoskeleton in nuclear import. *Adv Drug Deliv Rev* **55**, 761–771.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257**, 7847–7851.

Constantinescu, S. N., Cernescu, C. D. & Popescu, L. M. (1991). Effects of protein kinase C inhibitors on viral entry and infectivity. *FEBS Lett* **292**, 31–33.

Davis, P. D., Elliott, L. H., Harris, W. & 7 other authors (1992). Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. *J Med Chem* **35**, 994–1001.

Engh, R. A., Girod, A., Kinzel, V., Huber, R. & Bossemeyer, D. (1996). Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. *J Biol Chem* **271**, 26157–26164.

Hidaka, H. & Kobayashi, R. (1992). Pharmacology of protein kinase inhibitors. *Annu Rev Pharmacol Toxicol* **32**, 377–397.

Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.

Inagaki, M., Tanaka, T. & Hidaka, H. (1983). Calmodulin antagonists enhance calcium binding to calmodulin. *Pharmacology* **27**, 125–129.

Keay, S. & Baldwin, B. R. (1996). Evidence for the role of cell protein phosphorylation in human cytomegalovirus/host cell fusion. *J Gen Virol* **77**, 2597–2604.

Keenan, C. & Kelleher, D. (1998). Protein kinase C and the cytoskeleton. *Cell Signal* **10**, 225–232.

Kobayashi, E., Nakano, H., Morimoto, M. & Tamaoki, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159**, 548–553.

Krajcsi, P. & Wold, W. S. M. (1998). Viral proteins that regulate cellular signalling. *J Gen Virol* **79**, 1323–1335.

Loomis, C. R. & Bell, R. M. (1988). Sangivamycin, a nucleoside analogue, is a potent inhibitor of protein kinase C. *J Biol Chem* **263**, 1682–1692.

Lučin, P. & Jonjić, S. (1995). Cytomegalovirus replication cycle: an overview. *Period Biol* **97**, 13–22.

Lučin, P., Jonjić, S., Messerle, M., Polić, B., Hengel, H. & Koszinowski, U. H. (1994). Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J Gen Virol* **75**, 101–110.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* **76**, 1537–1547.

Miller, N. & Hutt-Fletcher, L. M. (1992). Epstein-Barr virus enters B cells and epithelial cells by different routes. *J Virol* **66**, 3409–3414.

Muranyi, W., Haas, J., Wagner, M., Krohne, G. & Koszinowski, U. H. (2002). Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina. *Science* **297**, 854–857.

Newton, A. C. (1997). Regulation of protein kinase C. *Curr Opin Cell Biol* **9**, 161–167.

O'Brian, C. A. & Ward, N. E. (1989). Binding of protein kinase C to N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide through its ATP binding site. *Biochem Pharmacol* **38**, 1737–1742.

Parekh, D. B., Ziegler, W. & Parker, P. J. (2000). Multiple pathways control protein kinase C phosphorylation. *EMBO J* **19**, 496–503.

Parker, P. J. & Murray-Rust, J. (2004). PKC at a glance. *J Cell Sci* **117**, 131–132.

Quick, J., Ware, J. A. & Driedger, P. E. (1992). The structure and biological activities of the widely used protein kinase inhibitor, H7, differ depending on the commercial source. *Biochem Biophys Res Commun* **187**, 657–663.

Redman, C., Lefevre, J. & Macdonald, M. L. (1995). Inhibition of diacylglycerol kinase by the antitumor agent calphostin C: evidence for similarity between the active site of diacylglycerol kinase and the regulatory site of protein kinase C. *Biochem Pharmacol* **50**, 235–241.

Root, C. N., Wills, E. G., McNair, L. L. & Whittaker, G. R. (2000). Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor. *J Gen Virol* **81**, 2697–2705.

Siecskarski, S. B. & Whittaker, G. R. (2002). Dissecting virus entry via endocytosis. *J Gen Virol* **83**, 1535–1545.

Slobbe-van Drunen, M. E. P., Vossen, R. C. R. M., Couwenberg, F. M. D., Hulsbosch, M. M., Heemskerk, J. W. M., van Dam-Mieras, M. C. E. & Bruggeman, C. A. (1997). Activation of protein kinase C enchances the infection of endothelial cells by human cytomegalovirus. *Virus Res* **48**, 207–213.

Smith, A. E. & Helenius, A. (2004). How viruses enter animal cells. *Science* **304**, 237–242.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007–1021.

Tamaoki, T. & Nakano, H. (1990). Potent and specific inhibitors of protein kinase C of microbial origin. *Biotechnology (N Y)* **8**, 732–725.

Toker, A. (1998). Signaling through protein kinase C. *Front Biosci* **3**, D1134–D1147.

Toullec, D., Pianetti, P., Coste, H. & 10 other authors (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**, 15771–15781.

Tugizov, S., Maidji, E., Xiao, J. & Pereira, L. (1999). An acidic cluster in the cytosolic domain of human cytomegalovirus glycoprotein B is a signal for endocytosis from the plasma membrane. *J Virol* **73**, 8677–8688.

Ward, N. E. & O'Brian, C. A. (1992). Kinetic analysis of protein kinase C inhibition by staurosporine: evidence that inhibition entails inhibitor binding at a conserved region of the catalytic domain but not competition with substrates. *Mol Pharmacol* **41**, 387–392.

Wathen, M. W. (2002). Non-nucleoside inhibitors of herpesviruses. *Rev Med Virol* **12**, 167–178.

Way, K. J., Chou, E. & King, G. L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21**, 181–187.

Wilkinson, S. E., Parker, P. J. & Nixon, J. S. (1993). Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J* **294**, 335–337.

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